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Several tumor suppressor genes (TSGs) have been cloned and found to be mutated in a variety of cancers, including breast cancer. However, few breast cancer-specific TSGs are known. The purposes of this proposal are to (1) clone novel TSGs specific to human breast cancer; (2) examine the alteration of these TSGs in primary breast tumors; and (3) identify their characteristics, regulation and function. We are utilizing the tetracycline (tet) regulable system. We have constructed a cDNA library from normal human breast epithelia and cloned this cDNA library into a vector that is negatively regulated by tet repressor (tetR) and simultaneously expresses enhanced green fluorescence. These vectors were then co-transfected into LCC6, 231, and MCF-7 cells that have the capability to express tetR. Upon withdrawal of tet, the repressed expression of the cDNA of interest is released, and the cDNA is expressed. Using a novel dye that was retained in nonproliferating cells, we were able to identify growth inhibited clones which were then sorted by Flow Cytometry. This functional screen has provided the basis for identifying TSGs which are expressed in the growth inhibited cells. Using PCR, we have obtained the insert sequences. We will now characterize these genes and begin to assess their function and expression in primary breast carcinomas.

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Introduction

Breast cancer is one of the most common malignancies of women in the United States. Most molecular genetic abnormalities contributing to breast cancer susceptibility remain unknown. Recent studies have revealed genomic changes in breast cancer, including amplification of proto-oncogenes, such as c-myc, c-erbB2, int-2, bcl-1, PRAD-1, EMS, EGF receptor (c-erbB 1), IGF-1 receptor, flg and bek (1,2) and intragenic mutations or suppressed expression in tumor suppressor genes (TSGs) including p53, Rb and p33 (3-5). TSGs constitute a relatively new class of genes and has been implicated in regulation of cell proliferation, cell cycle progression, apoptosis induction, and DNA repair and recombination. Many TSGs have been cloned from humans and found to be mutated in variety of human cancers (3-13). The study of TSGs should not only speed up basic cancer research, but it may also aid in the early diagnosis, prognostication, and treatment of human malignancies. Loss of heterozygosity (LOH), which is usually considered the hallmark of TSGs, has been observed in at least 15 out of the 23 pairs of chromosomes in human tumors. This result suggests that there may be numerous TSGs. However, only two genes specifically related to breast cancer have so far been cloned (BRCA-1) (14-16) or mapped to a specific chromosomal segment (BRCA-2) (17, 18); moreover the prevalence of intragenic somatic mutations in BRCA-1 is not very high in sporadic breast tumors (less than 10% of cases). Therefore, it seems likely that the cloning of new tumor suppressor genes of specific importance in breast cancer will be important and promising task for future research into this common disease.

The current proposal focuses on the isolation and characterization of novel TSGs in human breast cancer. A human epithelial eukaryotic cDNA expression library has now been constructed and transfected into the human breast cancer cell lines including MCF-7, MDA-MB-231 and MDA435/LCC6. Gene(s) inhibiting the growth of breast cancer cells and MCF-7 cells were considered candidate TSGs for breast cancer. These will be cloned and the full length cDNA sequence obtained. Expression of cloned genes will first be investigated in RNA populations derived from two immortalized "normal" human breast epithelial cell lines (A1N4 and MCF-10A), and in MCF-7 cells growth arrested either by antiestrogen-treatment or estrogen withdrawal. This approach provides a rapid and sensitive functional screen for growth inhibition-related activities using renewable resources, and is particularly important should a significant number of unique cDNAs be isolated. Subsequently, the expression of clones exhibiting an appropriate pattern of expression will be investigated in a series of RNA populations isolated from primary breast tumors. Once we have identified the most promising candidates, we will further screen genomic DNA from cell lines and primary breast tumors for somatic alterations, including deletion, mutation, and change in expression level. In the longer term, the most promising cDNAs will be studied to establish their characteristics and regulation. Putative TSGs that are growth-suppressive and specifically altered in breast cancers may be useful tools for the early diagnosis, prognostication, and eventual treatment of human breast cancers.

Body of Report

A. Brief statement of ideas and reasoning

Tumor suppressor genes (TSGs) function in normal tissues by regulating the growth of normal cells. Mutations, deletions, or other modes of inactivation of TSGs should contribute to

uncontrolled growth and malignant transformation of normal cells. Many TSGs have been cloned from humans and found mutated in variety of human cancers, including breast cancer (3-18). Many human chromosomes show high rates of loss of heterozygosity (LOH) in breast cancer; however, very few breast cancer-specific TSGs, such as BRCA-1, have actually been cloned (14). Moreover, the mutation rate of BRCA-1 in primary human breast tumors is less than 10% (15). Therefore, additional specific tumor suppressor genes for breast cancer are likely to exist. A cDNA expression library made from mRNA of normal human mammary glands should contain potential TSGs for human breast cancer, and thus can reasonably be used to isolate TSG(s) specific to breast cancer that inhibit the growth of breast cancer cells. **We propose a functional screen for the discovery of TSGs, which dramatically decreases the time to isolation and *a priori* demonstrates the function of novel TSGs. In addition, by using a cDNA expression library from normal human breast epithelia, transfected into breast cancer cells, we hope to clone TSG(s) that are specific to breast cancer.**

B. Hypotheses/Purposes

We hypothesize that:

1) Normal human mammary gland epithelia should contain all normally expressed potential TSGs for breast cancer.

2) TSGs play an important role in growth regulation of breast cancer cells in culture.

3) TSGs contribute significantly to the carcinogenic process in a significant portion of breast cancers.

The purpose of this proposal is to clone TSG(s) specific to breast cancer, examine their alteration in primary breast tumors, identify their characteristics, and ultimately study their regulation and function.

C. Technical Objectives

1). To clone novel TSGs for human breast cancer from a cDNA expression library made from normal human mammary gland epithelia.

2). To characterize the cloned TSGs by sequence homology analysis and study their functional effect on *in vitro* tumorigenesis for the most promising candidates.

3). In the long term: to study the regulation of cloned TSGs by finding their promoter regions and regulatory elements.

D. Experimental Methods, Assumptions and Procedures

Outline and rationale for approach

A major problem in the identification of growth inhibitory genes in a functional assay is that it is the non-proliferating (suppressed) cells that are the cells containing the genes of interest. We have constructed a novel approach that we believe is optimized for the specific purpose of identifying growth suppressor genes. Thus, we have utilized the tetracycline repressor (tetR)-based gene expression system. We have directionally cloned the cDNA library (see below) into an expression vector placing each cDNAs under the control of the tetracycline resistance operon that is regulated by tetR (19-22). These vectors were also able to express enhanced green fluorescence protein (EGFP) reporter by which the expression of genes of interest were monitored indirectly (22). These plasmids were co-transfected with the puromycin resistant plasmid into MCF-7 cells, MDA435/LCC6 cells and MDA-MB-231 cells already transfected with a plasmid expressing both the tetR repressor and the G418 resistance marker (MCF-$7^{tetR+neoR}$, $LCC6^{tetR+neoR}$, $231^{tetR+neoR}$). Upon withdrawal of doxycycline, the tetR/VP16 binds and activates transcription of the cDNA (19, 21). Double resistant and EGFP expressing cells were selected and expression of the gene of interest studied in the presence of increasing concentrations of doxycycline.

While we have a method to regulate genes expression, we also have an approach for enriching bulk transfected cell populations for growth inhibited cells. We have used our adaptation of the dye enrichment method of Maines et al. (23). The dye, PKH26-GL, (Sigma Chemical Co. St Louis, MO) (24) is non-toxic and specifically retained in non-proliferating cells. Since Flow Cytometry can be used to visually sort cells retaining dye (cells are maintained in the absence of tet), and there is a state-of-the-art Flow Cytometry Core Facility at the Lombardi Cancer Center, we were able to rapidly enrich the population for the growth inhibited cells, including cells that are completely growth arrested, sorting for the most red fluorescent cells (23,24). Thus, following the 24 hr recovery period immediately post-transfection, the cells were selected with puromycin, the resistance marker coexpressed in the plasmids containing the cDNA library. The concentration of puromycin was 1 μg/ml.

Surviving cell populations were stained with PKH26-GL and grown, now in the absence of tet, for the equivalent of several generations as described by Maines *et al.* (23). The estimated generation time for non-inhibited MCF-7 cells is 24-36 hrs and LCC6 and 231 18-24 hrs (25). Subsequently, single cells were aseptically sorted by Flow Cytometry (double sort - red for growth inhibition, green for gene expression) into the wells of 96-well plates. This provided individual cell clones expressing putative growth inhibitory genes. Cell clones containing growth suppressing cDNAs were then rapidly expanded by adding doxycyclineto block the putative TSG expression and release its growth suppression. The putative growth inhibitory genes were analyzed by PCR, subcloning and sequencing. Growth suppressor activity can be further confirmed, in a functional assay, by following experiments: (1) cloning putative tumor suppressing genes into expression vector; (2) transiently transfecting MCF-7 cells, LCC6 cells, and 231 cells with these vectors containing the genes interested; (3) observing cell growth by cell-cycle analysis using Flow Cytometry. RNA containing the expressed putative suppressor genes can be obtained, by introducing different concentrations of doxycycline to the culture medium to establish a tet-based dose response relationship for cell proliferation.

There are several significant advantages to this novel approach:

(1) Growth inhibition will be apparent only upon removal of tet, and this will reduce the background due to insertional mutagenesis, which could randomly produce slowly proliferating/growth inhibited cells independent of the inserted cDNA.
(2) We can identify genes that completely suppress proliferation, as well as genes that merely reduce the rate of proliferation.
(3) We can rapidly identify, in a functional assay, genes that specifically inhibit estrogen-regulated growth.

Previous work (reported in prior Progress Reports)
We have successfully completed most of the work envisioned in the original application. Thus, we have:

(1) successfully constructed cDNA libraries from reduction mammoplasties (normal human breast epithelia)

(2) converted the λ phage library into a plasmid cDNA expression library

(3) established stable MCF-7$^{tetR+neoR}$, MDA435/LCC6$^{tetR+neoR}$ and MDA231$^{tetR+neoR}$ cell lines

(4) begun to screen MDA231$^{tetR+neoR}$ cells for the expression of putative growth suppressor genes.

Shortly after the submission of last years report, Dr. Pu decided to take up a residency position at the University of Cincinnati. Recently, the mentor (Dr. Clarke) recruited a new Fellow (Dr. Yuelin Zhu) to complete these studies. A no-cost extension was only recently granted, along with a change in Fellow (to Dr. Zhu). Thus, the project has not been able to move forward substantially during the previous funding period. Nonetheless, Dr. Zhu (who started in Aug, 2000) is now poised to further study the clones isolated by Dr. Pu.

The putative growth inhibiting genes were identified by PCR with either TaqBead™ Hot Start Polymerase (Promega, Cat#M5661), or Expand™ High Fidelity PCR System (Boehringer Mannheim, Cat#1 732 650) using the genomic DNAs as templates, which were extracted from the cell clones containing growth suppressing genes. The PCR primers were designed containing partial sequences of pBI-EGFP, one pair # 426 (5'-GTACCCGGGTCGAGTAGGCGTGTA-3') and # 650 (5'-GGTCCCCAAACTCACCCTGAAGT-3'), and another pair # 426 and # 657 (CAATCAAGGGTCCCCAAACTCACC-3'), according to primer design programs (DNAStar). We isolated several PCR products were found and ranged from 600 bp to 2 kb.

The PCR segments were cut out from the gels and purified. The purified cDNAs were then re-amplified with the same primers. The obtained cDNA products were sequenced either by direct sequencing or subcloning the cDNAs into the vector and then sequencing. We have recently been able to identify one 700 bp sequence as being located on chromosome 9 (36-38). The precise location is unknown but under investigation. Several putative suppressor genes have been implicated, through the presence of LOH, on this chromosome in human breast tumors.

F. Key Research Accomplishments (Progress on the Statement of Work)
In the past months, we have successfully followed our previous plans as described below.

Technical Objective 1: Identify putative TSG(s)

Task 1: Months 1-6: Construct and Characterize cDNA library - **completed** (this took almost 12 months to complete since Dr. Pu was not the original awardee but began the work several months after the initial award - the original awardee did not take up the award and a personnel change was approved by USAMRMC).

Task 2: Months 6-8 (took 12-18): Re-construct plasmid cDNA library and transfect MCF-$7^{tetR+neoR}$ cells, $LCC6^{tetR+neoR}$ cells, and $231^{tetR+neoR}$ cells - **completed** (delayed by the cDNA library construction).

Task 3/4: Months 8-24 (now 18-36): Identify cells containing growth inhibitory genes and clone TSG(s) - **growth inhibited cells have been cloned; several putative TSGs (PCR products) also have been cloned.**

Technical Objective 2: Characterize putative TSG(s)

Task 5: Months 24-36: cDNA sequencing and sequence analysis - **in progress.**

Task 6: Months 30-48*: Screen tumors for mutations in putative TSG(s) - **in progress.**

*We anticipate that completion of these studies will take longer than the three year period. However, it is likely we have sufficient data to enable the Fellow to apply for additional funding.

We should mention that up-to-now, we have not had any special problems in accomplishing any of our tasks. In the near future (next few months), we will focus our attention on DNA sequencing, sequence analysis and will start functional assays to transfect these putative TSGs into human breast cancer cell lines. We also will screen tumors for mutations in putative TSG(s), as indicated in the work statement.

We have successfully constructed and performed our initial characterization of the first library from a reduction mammoplasty (normal breast tissue). We also have obtained clones of MCF-7 cells MDA435/LCC6 cells, and MDA-MB-231 cells, transfected with the tetR vector. With the cloning of pBI-EGFP cDNA expression library, the initial transfection of MCF-$7^{tetR+neoR}$ cells **MDA435/LCC6$^{tetR+neoR}$ cells and MDA-MB-231$^{tetR+neoR}$** ***cells*** *were successfully completed. Our accomplishments of identifying cell clones containing putative growth inhibitory genes have provided a solid basis. Current DNA sequencing and analysis have lead us so close to the final cloning TSGs as described in original application.*

Since the initial award, we have had several delays, none of which were scientific in nature. The original applicant (Dr. Lei) did not take up the award. Dr. Pu, who performed most of the studies to-date, gave birth in the second year and subsequently left to take up a clinical residency at the University of Cincinnati). Only in the last few weeks has a third candidate joined the laboratory to complete the studies originally envisioned for year 3. Nevertheless, we remain

on-track with regard to the science, and should be able to complete many of the proposed studies in the no-cost extension that was recently granted. We believe the data to be highly encouraging, and indicative of the nature of the training environment, the commitment of the mentor and our ability to perform the remainder of the proposed studies within the anticipated time frame.

Other Training

In addition to the training obtained in the laboratory, Dr. Zhu will participate in several other research activities within The Lombardi Cancer Center. Dr. Zhu will attend the regular center-wide research Journal Club, and is required to present 1-2 times per year. He also will attend and participate in the center-wide Research Data Meetings, at which he also is required to present 1-2 times per year. Dr. Zhu will interact with the other scientists within Dr. Clarke's laboratory and to work and consult with other investigators within the Cancer Center. Dr. Zhu will be expected to attend the AACR and USAMRMC meetings next year and to submit abstracts for her data. He also may attend other meetings as necessary or appropriate.

Future Plans

Each transfected clone will initially be examined for the integrity and copy number of transfected cDNAs by Southern analysis (there may be more than one plasmid in some transfectants), and the appropriate mRNA expression by Northern analyses. The Northern analysis will provide critical information on the size of the expressed transcript(s). Since we have used a regulable promoter approach, we will perform dose response analyses with increasing concentrations of the regulating agent (i.e., tet). This will enable us to assess the potency of the gene, i.e., what level of expression is associated with a corresponding level of growth inhibition. The effects of doxycycline on expression of mRNA from the repressed promoter will be monitored by Northern analysis. Controls will consist of parallel cultures of non-transfected cells and cells transformed with the tetR operator expression vectors without the cDNA inserts and that are treated with tet.

We cannot exclude the possibility that the level of expression required for growth suppression is below the limit of detection by Northern. When this occurs we will use RNase protection or semiquantitative PCR to detect product. We will use primers from the portion of the regulable promoter sequence that is transcribed in the final product, and a site internal to the inserted cDNA sequence. This also will enable us to distinguish those products amplified from newly transcribed RNA from those derived from the endogenous gene.

Reportable Outcomes

Pu, L.-P., Skaar, T.C., Leonessa, F. **& Clarke, R.** "Tumor suppressor genes in breast cancer". *DOD Breast Cancer Research Program* pp108, 2000.

This was presented by Dr. Clarke, since Dr. Pu had already left the laboratory.

Conclusions

This is a postdoctoral fellowship application by an individual who previously worked in another field, and was not the original recipient of the award. Despite having lost considerable time for

reasons unrelated to the project, we have maintained the scientific direction and approach completion of the original goals.

We have now successfully constructed the cDNA library and performed our initial characterization of the first library from a reduction mammoplasty (normal breast tissue). We also have obtained two clones of MCF-7 cells transfected with the tetR vector. With the cloning of pBI-EGFP cDNA expression library, we have successfully transfected MCF-$7^{tetR+neoR}$ cells, $LCC6^{tetR+neoR}$cells, and $231^{tetR+neoR}$ cells with pBI-EGFP cDNAs. We have identified cell clones contained putative growth inhibitory genes and sequenced some of candidates for putative growth inhibitory genes, providing a basis for the final cloning of TSGs as described in our original application. Furthermore, her participation in local and national meetings permits a further level of training and exposure to breast cancer research. We have also presented this work at the AACR meeting in 1999. Thus, we believe that we are making excellent progress towards the successful accomplishment of the aims and goals of the original application.

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Appendices

Dr. Yuelin Zhu's CV **(Dr. Zhu joined the mentor's laboratory Aug 1, 2000).**

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1995-1998 Lecturer in General Surgery and Surgical Oncology,
Department of Surgery, Medical School of Shanghai Tiedao University, Shanghai.

HOSPITAL APPOINTMENTS

1995-1998 Attending Surgeon in General Surgery and Surgical Oncology, Surgery Branch,
the Affiliated Hospital of Shanghai Tiedao University, Shanghai.
1988-1992 Surgeon in General Surgery, Surgery Branch,
Funing Hospital, Jiangsu.

TEACHING EXPERIENCE

1995-1997 Lecturer, Department of Surgery,
Medical School of Shanghai Tiedao University, Shanghai
Taught General Surgery, Surgical Oncology and Molecular Pathology.

RESEARCH EXPERIENCE

1998-1999 National Cancer Institute, National Institutes of Health.

1. **Methylation status of multiple genes (p16, E-Cadherin, ER, RAR beta, MGMT, hMLH1) in ER negative breast cancer.**
2. **The anti-cancer effect on ER negative breast cancer by demethylation drugs (5-aza-deoxycitidine, TSA, etc.).**
3. **The cell cycle and apoptotic effect of combinations of retinoids (all-trans, 9c, 4-HPR) and antiestrogens on breast normal , high risk and cancer epithelia.**

Major techniques: Methylation-specific PCR, quantitative RT-PCR, Sequencing, Northern, Southern, Western Blot, ligand-binding immunopreciptation assay.
Cell Culture: HMEC(AG11134, AG11132), high-risk breast epithelium cell lines (Standard and Addeton) and ER+/- breast cancer cell lines.

1995-1998 Laboratory of Cancer Research, Medical School of Shanghai Tiedao University, Shanghai

Multiple molecular genetic alterations in gastrointestinal carcinomas.
Study of tumor suppressor gene p16 and nm23 in gastric carcinoma. Expression of c-erbB-2 and EGER in gastric cancer. Major techniques: Inmmunohistochemical techniques, pathological techniques, Flow Cytometry and In situ hybridization.

1992-1995 Department of Surgery, Shanghai Second Medical University, Shanghai.

1. Quantitation of DNA and RNA content by Image Cytometry.
Methyl Green-Pyronine staining in Image Cytometry quantitation of DNA and RNA content in gastric cancer, using the techniques of Image Cytometry, histochemistry and pathology.

2. **p53 and DNA ploidy in gastric cancer.**
Major techniques: Image Cytometry, Inmmunohistochemical techniques, pathological techniques, Flow Cytometry, PCR and In situ hybridization.

RESEARCH INTEREST

Cell cycle control
Apoptosis pathways
Methylation
Molecular biology of breast cancer and gastrointestinal cancer
Retinoids and antiestrogens

MAJOR TECHNICAL TRAINING

Well trained with major techniques of pathology, cellular and molecular biology, including DNA, RNA and protein analysis.

NIH-FAES Biotechnology Training Classes:

1. Polymerase Chain Reaction and Molecular Hybridization Technology (TRAC 9). 1999.
2. Cell Cycle: Principle and Methods (TRAC 13M). 1999.
3. DNA replication and transcription. (2 credits, 20 weeks). 1998.
4. Advanced English conversation (ENGL 304). 1998.

Shanghai Second Medical University
Molecular Pathology Training. 1994

Publications

1. Yuelin Zhu, et al., 1995, Functional studies of p53 and DNA ploidy in Gastric cancer. Shanghai Med., 19(3):135.
2. Yuelin Zhu, et al., 1996, Multiple genetic expression abnormalities in gastric cancer. Chin. J. Onco., 18(3):199.
3. Yuelin Zhu, et al., 1996, Prognostic significance of multiple genetic abnormalities in gastric cancer, Chin. J. Clin. Onco., 23 suppl.:108.
4. Yuelin Zhu, et al., 1996, Clinicopathological and prognostic significance of multiple molecular genetic abnormalities in gastric cancer. Acta Universitatis Medicinalis Secondae, Shanghai, 16(4):233.
5 Yuelin Zhu, et al., 1996, Simultaneously quantitation of the content of DNA and RNA in the cell of gastric cancer using Methyl Green-Pyronine staining procedure. Acta Universitatis Medicinalis Secondae, Shanghai, 17(1):25.
6 Yuelin Zhu, et al., 1996, Improvement of Methyl Green-Pyronin staining procedure. Chin. J. Histochem. and Cytochem., 5(2):227.
6. Yuelin Zhu, et al., 1996, Improvement of Image Cytometry in the studies of gastrointestinal carcinoma. Shanghai Med., 20(2):56.
7. Yuelin Zhu, et al., 1997, Functional studies of ras and EGFR expressions in gastric cancer. J. Surgery, 1(3):86.
8. Yuelin Zhu,et al.,1997, Expressions of c-erbB-2 and EGFR in gastric cancer. Chin. J. Clin. Onco.,23(3):855.
10. Yuelin Zhu: Chapter 27 Application of quantitative pathological technique in study of gastrointestinal tumor. In: Genjing Lin. ed. New concept of Gastroenterology. Shanghai: Shanghai Medical University Press, 1997:361.
11. Multiple gene demethylation effect and apoptotic effect of 5-aza-deoxycitidine and Trichostatin A on MB-MDA-231. Submitting, 1999.

AWARDS AND HONORS

1997 Overseas Study Scholarship from the China Scholarship Council (CSC)
1994 Best Thesis Honor in Shanghai Second Medical University
1988 Distinguishing Medical Student in Nantong Medical College

REFERENCES

Ruinian Wang, M.D., Professor, Department of Pathology, Shanghai Second Medical University, 227 Chongqing Nan Road, Shanghai 200025, P.R. China. Tel: (8621)638-46590X pathology.

Yichu Zhang, M.D., Professor and Director, Department of Surgery, Shanghai Second Medical University, 1665 Kongjiang Road, Shanghai 200092, P.R. China. Tel: (8621)654-54630X surgery.

Sihui Ye, M.D., Professor and Director, Department of Surgery, Medical School of Shanghai Tiedao University, 301 Yanchang Road, Shanghai 200072, P.R. China. Tel: (8621)567-70588Xsurgery.